Critical Role of Glutamine 252 in the Hormone-Dependent Transcriptional Activity of the Thyroid Hormone β1 Nuclear Receptor

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ABSTRACT: To understand the molecular basis of the ligand-dependent transcriptional activity of thyroid hormone nuclear receptors (TRs), we investigated the effect of mutation of glutamine 252 (Q252) on the function of human TR subtype β1 (wTRβ1). Q252 is conserved in TRs in all species and is located in a region of the hormone binding domain that has been shown to undergo 3,3′,5-triiodo-L-thyronine (T_3) induced conformational changes. Q252 was mutated to Gly (Q252G) or Asn (Q252N) and their immunoreactivity, hormone, and DNA binding activities were characterized. Mutants Q252G and Q252N bound to T_3 with similar affinity as the wTRβ1. However, they failed to interact with a monoclonal anti-wTRβ1 antibody whose epitope is located in the region of amino acids 248−256, suggesting that mutation of Q252 to Gly or Asn resulted in local structural alteration in TRβ1. In addition, mutation of Glu to Gly or Asn led to increases in their binding to the thyroid hormone response elements (TREs) as homodimers and as heterodimers with the retinoid X receptor. Mutants Q252G and Q252N were more effective as repressors in the absence of T_3, while both had a 1.5−2-fold higher T_3-dependent transcriptional activity mediated by three TREs than the wTRβ1. The increases in the transcriptional activity were not due to an increase in the expression of the mutant receptor proteins because the *in vivo* expression level of the mutant receptor proteins was identical to that of the wTRβ1. Our data indicate that the T_3-dependent transcriptional activity is not entirely dependent on the T_3 binding activity of the receptor. The interplay of ligand and DNA binding domains plays a pivotal role in the transcriptional activity of the TRs.

Human β1 thyroid hormone receptor (TRβ1)[1] is a member of the steroid hormone/retinoid acid receptor superfamily of homologous transcription factors, which regulate gene expression upon binding small hydrophobic ligands. From sequence considerations, this protein has been divided into four distinct domains: domain A/B (residues 1−106), the DNA binding domain C (107−175), a hinge domain D (176−243), and the ligand binding domain E (244−461). Domain C recognizes specific response elements in the DNA sequence, while domain E, besides binding ligand, participates in transcriptional activation and repression (*1*, *2*). Recent studies have revealed a more complex situation. Using deletion mutants, we showed that residues K206−D216, located as part of domain D, are necessary for the folding of the ligand binding domain to an active form (*3*, *4*). Binding of hormone to intact TRβ1 renders the receptor molecule less susceptible to proteolysis. The region that is protected from proteolysis as a result of hormone binding is S207−F245 (*5*). Furthermore, monoclonal anti-TRβ1 antibody C3, whose epitope is localized to the region of E248−V256, competitively inhibits the binding of the receptor to the thyroid hormone 3,3′,5-triiodo-L-thyronine (T_3) (*6*). These observations suggested that this region of the molecule is involved in the hormone binding site. Wagner et al. (*7*) have recently determined the crystal structure of a fragment of rat TRα1, containing domains D and E, complexed with a thyroid hormone analog, 3,5-dimethyl-3′-isopropyl-L-thyronine (Dimit). In agreement with our findings, they found only one structural domain which bound Dimit, corresponding to TRβ1 residues K211−D461. However, they specifically reported that residues K211−V256 did not directly contact the bound Dimit and that residues K244−D265 formed the most flexible part of the molecule.

Based on the known structures of three ligand binding domains (LBDs) of the holo-RARγ, the apo-RXRα, and the holo-TRα1, a common fold and basis for transcriptional activation by ligand has been proposed (*8*, *9*). The LBD is folded as an antiparallel α-helical sandwich, containing 11−12 α-helices and 2−4 short β-strands. The ligand binds in a cavity, completing the middle layer of the sandwich. A major conformational change accompanies ligand binding. In apo-RXRα, the C-terminal helix H12, which contains a transcriptional activation function, AF-2, common to all nuclear receptors, sticks out from the main body of the molecule. It is held in place by interactions with residues

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[1] Abbreviations: T_3, 3,3′,5-triiodo-L-thyronine; TR, thyroid hormone receptor; TRβ1, human TR subtype β1; RXRβ, human retinoid X receptor, subtype β; RARγ, retinoic acid receptor, subtype γ; TRE, thyroid hormone response element; mAb, monoclonal antibody; SDS−PAGE, sodium dodecyl sulfate−polyacrylamide gel electrophoresis; EMSA, electrophoretic mobility shift assay; F2, chicken lysozyme thyroid hormone response element (an everted repeat of the half-site binding motifs separated by six nucleotides); DR4, direct repeat of the half-site binding motifs separated by four nucleotides; Pal, palindromic thyroid hormone response element; ME, thyroid hormone response element of the rat malic enzyme gene; LBD, ligand binding domain; DBD, DNA binding domain.

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Table 1: TR Sequences around Ω-Loop Region (H, α-Helix; s, β-Strand)

		--H2→<s2>		←H3--
Rat, Human[a]	α1	^{186}KQRRKFLPDD	IG**Q**SPIVSMP	DGDKVDLEAF^{215}
Xenopus[a]	αA	--------E-	-----MA---	----------
Xenopus[a]	αB	--------E-	-----MA---	----------
Chicken[a]	α1	--------E-	-----MA---	----------
Ovine[b]	β1	--K-----E-	---A---NA-	E-G-------
Xenopus[a]	βA	--K-----E-	---A---NA-	E-G-------
Xenopus[a]	βB	--K-----E-	---A---NA-	E-G-------
Rat	β1	--K-----E-	---A---NA-	E-GQ------
Human	β1	^{240}K-KP----E-	---A---NA-	E-G-------^{269}
Human[c]	RXRα	^{253}NMGLNPSS	PNDP^{264}	
		[Omega-loop]		

[a] Sequence is from Yaoita *et al.* (1990). [b] Sequence is from Tucker and Polk (1996). [c] Sequence is from Bourguet *et al.* (1995).

in an Ω-loop between helices H2 and H3 (*10*). In the structures of RARγ and TRα1 LBDs, which were crystallized in the presence of ligand, H12 is folded back onto the surface of H3 and H4, while the Ω-loop has flipped over (*7, 11*). These motions seal the filled ligand binding cavity and move the region that is involved in ligand-dependent transactivation function (AF-2), either making it unavailable for interactions with other molecules, which may participate in the transcriptional apparatus, or creating a new surface for this binding.

However, the effects of this conformational change on the other properties of the intact receptors are unknown. At present, nothing is known of the structure of the unliganded TR LBD or how the dynamic and structural changes accompanying binding can influence the properties of the other regions of the receptor molecule. It seems likely that the flexible region situated between the hormone and DNA binding domains (e.g., amino acids K244−D265 of TRβ1) must be involved in interactions between them and in the hormone-dependent regulation of their biological properties. The interactions between H12 and the Ω-loop are well-defined in apo-RXRα. Transcriptionally essential residue F450 is close to the N-terminus of H3, and E453 is hydrogen-bonded to N262. The presence of a similar structure has been suggested to occur in apo-TRs (*8, 9*) even though the X-ray crystallographic structures are not yet available.

Because of the importance of the dynamic changes of Ω-loop in the ligand-dependent transcriptional activation of TRs, we sought to identify the critical role of the amino acids in the Ω-loop. Our strategy was based on the following considerations. The sequence between H2 and H3 is much longer in TRs (23 residues) than in RXRα (12 residues, see Table 1). The first few residues of this sequence form a short β-strand and the second half of the sequence is highly variable in composition, while the sequence of the AF-2 in TRs is absolutely conserved (*12*). Anticipating that regions which interacted with AF-2 would also be conserved, we focused on the region P247−P254 (PEDIGQAP) in TRβ1 as a suitable candidate for its Ω-loop and specifically on the nonvariant Q252 as a hydrogen-bond donor. We chose to mutate Q252 to Gly so that there was no possibility of hydrogen bonding and to Asn to ascertain the spatial requirement. Mutation of Gln to Gly or Asn produced important changes in the activities of TRβ1 and thus Q252 is critically involved in the transcriptional activation of TRβ1.

MATERIALS AND METHODS

[α-^{32}P]dCTP (3000 Ci/mmol), L-[^{35}S]methionine (10 mCi/mL), and ECL Western blotting kit were obtained from Amersham Life Science Inc. (Arlington Heights, IL). [^{125}I]T_3 (2206 Ci/mmol) was from Du Pont−New England Nuclear (Boston, MA). TNT coupled reticulocyte lysate system and restriction enzymes were from Promega (Madison, WI). Lipofectamine reagent was purchased from Life Technologies (Gaithersburg, MD).

Construction of Q252G and Q252N Mutant Expression Plasmids. The mutations were introduced into the parental vector pCJ3 using polymerase chain reaction (PCR). pCJ3 is a T7 expression plasmid encoding wTRβ1 (*13*). A unique restriction site for *Eco*RV was introduced in the 5′ primer for screening purposes. The sequence of antisense primer for Q252G is 5′ AAC CTT TCC ACC TTC TGG GGC ATT GAC TAT TGG TGC TCC TCC GAT ATC TTC TGG CAG GAA TTT CCG 3′ (nucleotides 1077−1012) and for Q252N is 5′ AAC CTT TCC ACC TTC TGG GGC ATT GAC TAT TGG TGC GTT TCC GAT ATC TTC TGG CAG GAA TTT CCG 3′ (nucleotides 1077−1012). The numbering of the nucleotide sequence is according to Weinberger et al. (*14*). The sequence of sense primer is 5′ TAA TAC GAC TCA CTA TAG GGA 3′. The cloning was completed in two steps. First the PCR products were cloned into a TA vector (Invitrogen, San Diego, CA). The pCJ3 plasmid and the TA clones containing the PCR fragments were restricted by *Nde*I and *Pfl*MI. The *Nde*I- and *Pfl*MI-released fragments from the TA clones were then religated onto the linearized pCJ3 vector containing the compatible ends. The pCJ3 vectors containing the Q252G

and Q252N mutations in the wTRβ1 cDNA were termed pCJ3-Q252G and pCJ3-Q252N, respectively.

The mammalian expression plasmids for the mutants were derived from the wTRβ1 expression vector pCLC51 (*15*), which uses cytomegalovirus promoter. pCLC51, pCJ3-Q252G, and pCJ3-Q252N plasmids were restricted by *Sma*I and *Pst*I. The fragments released by *Sma*I and *Pst*I digestion of pCJ3-Q252G and pCJ3-Q252N were religated into pCLC51 vector containing the compatible ends to give pCLC51-Q252G and pCLC51-Q252N, respectively. The presence of desired mutations in the plasmids was verified by restriction mapping and confirmed by direct DNA sequencing.

T_3 Binding Assay. Wild-type or mutant (3 μL) TRβ1 prepared by the *in vitro* transcription/translation using TNT coupled reticulocyte lysate system according to the manufacturer's instructions was incubated with 0.2 nM [^{125}I]T_3 in the absence or presence of increasing concentrations of unlabeled T_3 in buffer B (50 mM Tris-HCL, pH 8.0, 0.2 M NaCl, 10% glycerol, 0.01% Lubrol, and 1 mM dithiothreitol). After incubation of the mixture (0.266 mL) at 20 °C for 1 h, the [^{125}I]T_3-bound TRs were separated from the free [^{125}I]T_3 by passing the mixture through a Sephadex G-25 column (bed volume, 3 mL) as described previously (*3*). The binding data were analyzed by using eq 1 based on direct competition between [^{125}I]T_3 and the unlabled T_3 for a single site on the receptor. The concentration of radioactive complex is given by

$$[\mathrm{Rh}] = \frac{[\mathrm{R}]_0[\mathrm{h}]}{K_\mathrm{d} + [\mathrm{h}] + [\mathrm{c}]} \quad (1)$$

where $[\mathrm{R}]_0$ is the total concentration of receptor, [h] and [c] are the concentrations of [^{125}I]T_3 and the unlabeled T_3, respectively, and K_d is the dissociation constant of the hormone−receptor complex. The data were fitted directly to eq 1 using the PC-MLAB program (Civilized Software, Bethesda, MD), to evaluate K_d and $[\mathrm{R}]_0$.

Immunoprecipitation. The ^{35}S-labeled wild-type and/or mutant TRβ1 proteins synthesized by using the TNT system were incubated with increasing amounts of three different anti-TRβ1 mAbs, C3 (*6*), C4 (*16*), and J51 (*17*), for 45 min at room temperature. After incubation, the antigen−mAb complex was absorbed by Staph A, which was preincubated with rabbit anti-mouse heavy and light chains. The immunoprecipitates were analyzed by SDS−polyacrylamide gel electrophoresis and autoradiographed.

Electrophoretic Mobility Shift Assay. Two complementary oligonucleotides containing F2, DR4, or Pal sequences (*18*, *19*) were annealed, and the recessed 3′ end were filled in with 5′-[α-^{32}P]deoxyCTP (100 μCi) using DNA polymerase (Klenow fragment). The labeled probes were purified from a 12% polyacrylamide gel as previously described (*18*). The concentration of the labeled probes was determined by measuring the absorption at 260 nm in a spectrophotometer. The specific activities of the labeled DNA probes were determined by counting an aliquot in a β-counter and expressed as microcuries per femtomole. Wild-type and mutant TRβ1 were synthesized by using TNT-coupled reticulocyte lysate system. The amounts of synthesized receptors were determined by trichloroacetic acid precipitation of ^{35}S-labeled receptor proteins and by SDS−PAGE followed by quantification by PhosphoImager. Concurrently, unlabeled wild-type and mutant TRβ1 were also synthesized. Equal amounts of unlabeled receptor proteins were used in binding to TREs.

Transient Transfection Assay. CV1 cells (3×10^5 cells/60-mm dish) were plated 24 h before transfection in Dulbecco's modified Eagle's medium (DMEM) containing 10% fetal bovine serum (FBS). Cells were transfected with CAT reporter plasmids (pTK28mCAT for Pal TRE, pMETKCAT for DR4 TRE, or pF2TKCAT for F2 TRE; each 0.4 μg) and 0.2 μg of wTRβ1 or mutant receptor using the lipofectamine transfection method according to the manufacturer's procedure (Life Technologies, Gaithersburg, MD). After 6 h, the transfection medium was replaced with fresh DMEM containing 10% FBS and the cells were incubated overnight. Next day the cells were washed with phosphate-buffered saline (PBS) and grown in fresh DMEM containing 10% thyroid hormone-depleted FBS. Fifteen hours before cells were harvested, T_3 (100 nM) was added to appropriate dishes. Cells were lysed, and CAT activity was determined using equal volumes of total lysate (*20*). CAT activity was corrected for the equal amount of the lysate proteins.

Detection of Receptor Protein Expression by Western Blotting. Twenty micrograms of the whole cell lysate proteins prepared from the transfected and untransfected CV1 cells were electrophoresed on an SDS−10% polyacrylamide gel. The resolved proteins were transferred onto 0.45 μm nitrocellulose membrane and the membrane was blocked with Tris-buffered saline containing 5% nonfat dried milk. The membrane was probed with 1 μg/mL TR mAb C4 (*16*). After incubation with the primary antibody, the membrane was washed with Tris-buffered saline containing 0.1% Tween 20 and then incubated with horseradish peroxidase-conjugated secondary antibody. The protein bands were detected by the enhanced chemiluminescence technique according to the manufacturer's directions (ECL Western blotting detection reagents, Amersham Life Science, Arlington Heights, IL). The protein bands were visualized by autoradiography.

RESULTS

Mutation of Q252 to G or N in TRβ1 Did Not Affect Its T_3 Binding Activity. To evaluate the role of amino acids in the Ω-loop region of TRβ1 in the context of intact receptor, we chose to mutate Q252 to G and N. As stated in Materials and Methods, the identity of mutants was confirmed by restriction map analysis and direct DNA sequencing. We further characterized the mutants by comparing their molecular weights with that of wild-type TRβ1 (wTRβ1) using the TR proteins prepared by *in vitro* transcription/translation. As shown in lanes 2 and 3 of Figure 1, two molecular species with molecular weights of 55K and 52K were detected for mutants Q252G and Q252N, which were similar to those seen for wTRβ1 (lane 1 of Figure 1). The smaller proteins with molecular weight of 52K were derived from translation initiated from a downstream ATG (*14, 21*). These results indicate that intact mutant proteins were expressed.

Using the wild-type and mutant TRs shown in Figure 1, we examined their T_3 binding activity by competitive binding assay. As shown in Figure 2, the displacement curves from mutants Q252G and Q252N were indistinguishable from that of wTRβ1. The binding data were analyzed and K_ds were calculated to be 0.33 ± 0.04 and 0.26 ± 0.07 nM for Q252G and Q252N, respectively. These K_ds were similar to that of wTRβ1 (0.34 ± 0.14 nM), indicating that changing glutamine 252 to either G (Q252G) or N (Q252N) did not alter T_3 binding affinity of wTRβ1.

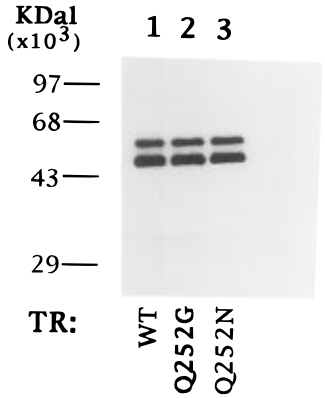

FIGURE 1: Autoradiograms of the wild-type and mutant TRβ1 proteins expressed by *in vitro* transcription/translation. Two microliters of ^{35}S-labeled protein synthesized by *in vitro* transcription/translation was loaded onto SDS−10% polyacrylamide gel. The gel was dried and autoradiographed.

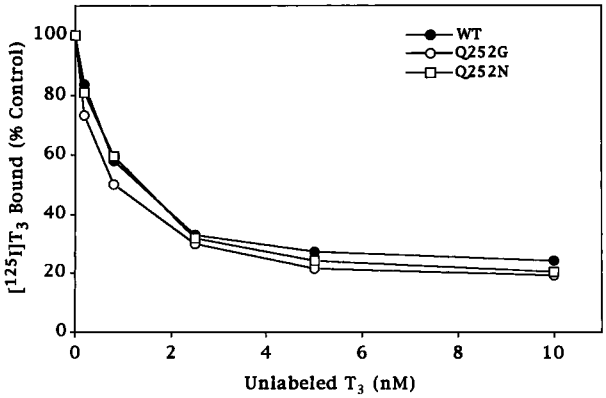

FIGURE 2: Competitive binding of the wild-type and mutant TRβ1 to [^{125}I]T_3. Equal amounts of the *in vitro* translated wTRβ1 (●), Q252G (○), and Q252N TRβ1 (□), were incubated with 0.2 nM [^{125}I]T_3 in the absence or presence of increasing concentrations of unlabeled T_3. The free and bound [^{125}I]T_3 were separated as described in Materials and Methods. Data are expressed as percentage of [^{125}I]T_3 bound in the absence of unlabeled T_3.

Change of Q252 to G or N Led to Loss of Immunoreactivity to mAb C3. We have recently developed an anti-wTRβ1 monoclonal antibody C3 (mAb C3) whose epitope has been mapped to E248−V256 (*6*). Using mAb C3 as a tool, we probed the possible conformation changes due to mutation of Q252. As a control, we also used two other anti-wTRβ1 mAbs, J51 (*17*) and C4 (*16*), whose epitopes are located on the amino and carboxyl termini of wTRβ1, respectively. We immunoprecipated [^{35}S]methionine-labeled *in vitro*-translated wild-type and mutant TRβ1 proteins with mAbs C3, C4, and J51. Figure 3 compares the autoradiograms of the immunoprecipitated wTRβ1 (panel A) and mutant Q252G (panel B). Lane 1 in each panel of Figure 3 shows the [^{35}S]methionine-labeled wTRβ1 as a standard marker. wTRβ1 (Figure 3A), as expected, was immunoprecipitated by mAb C3 (lanes 2−5), mAb C4 (lanes 6−9), and mAb J51 (lanes 10−13) in a concentration-dependent manner. The immunoprecipitated wTRβ1 were specific because increasing concentrations of an irrelevant mAb did not immunoprecipitate wTRβ1 (lanes 14−17). In contrast to the wTRβ1, mAb C3 did not immunoprecipitate mutant Q252G (lanes 2−5, Figure 3B) at any concentration. However, mAbs C4 (lanes 6−9, Figure 3B) and J51 (lanes 10−13, Figure 3B) immunoprecipated Q252G similarly as for wTRβ1 (lanes 6−13 of Figure 3B vs lanes 6−13 of Figure 3A). We also examined the immunoreactivity of

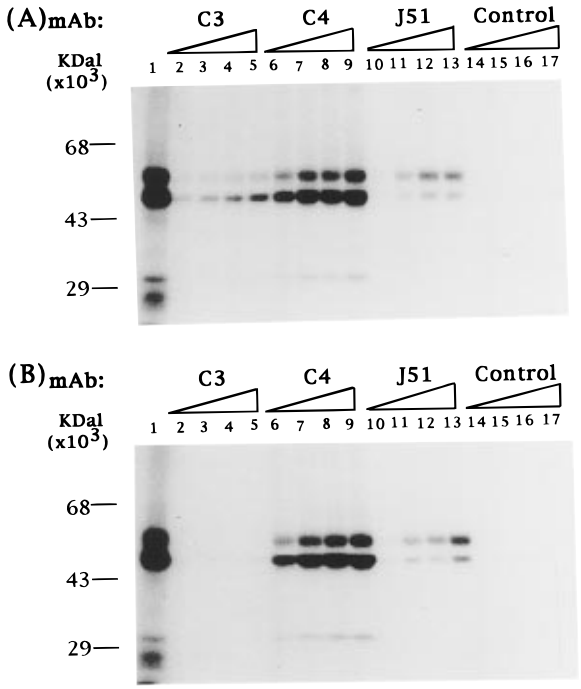

FIGURE 3: Autoradiograms of the wTRβ1 and mutant Q252G immunoprecipitated by mAb C3, C4, and J51. (A) Four microliters of ^{35}S-labeled wTRβ1 was immunoprecipitated with increasing concentrations (25, 100, 250, and 500 ng) of mAb C3 (lanes 2−5), mAb C4 (lanes 6−9), mAb J51 (lanes 10−13), or a control antibody (lanes 14−17). Lane 1 shows the standard marker using the *in vitro* translated wTRβ1 (1 μL) without immunoprecipitation. (B) Four microliters of ^{35}S-labeled mutant Q252G was immunoprecipitated with increasing concentrations of mAb C3 (lanes 2−5), mAb C4 (lanes 6−9), mAb J51 (lanes 10−13), or a control antibody (lanes 14−17). For comparison, lane 1 shows the standard marker from the *in vitro* translated Q252G (1 μL) without immunoprecipitation.

mutant Q252N with the same three mAbs and found that Q252N also failed to be recognized by mAb C3 but had the same immunoreactivity with mAb C4 and J51 as the wTRβ1 (data not shown). These results indicate that mutation of Q252 to G or N affected local structure but had no global effect on the structure of TRβ1.

Enhancement of in Vitro DNA Binding Activity of wTRβ1 by Changing Q252 to G or N. To evaluate whether Q252 plays a role in the DNA binding activity of wTRβ1 either as a homodimer or as a heterodimer with RXRβ, we compared the DNA binding activity of wTRβ1, Q252G, and Q252N mutants to F2, in which the half-site binding motifs are arranged in an everted repeat separated by six nucleotides. Figure 4 shows a representative autoradiogram of the binding of wTRβ1 (lanes 2−7) and mutants Q252G (lanes 8−13) and Q252N (lanes 14−19) to increasing concentrations of [^{32}P]-labeled F2. Inspection of the intensities of the homodimeric bands in Figure 4 indicate that, at each given F2 concentration, more mutants were bound to F2 than to the wTRβ1 (e.g., lanes 8 and 14 vs lane 2). The intensities of the bands were quantified and the binding data were analyzed, which gave apparent K_ds of 132 ± 9.7, 54.4 ± 9.2, and 37.1 ± 7.8 nM for the binding of wTRβ1 and mutants Q252G and Q252N to F2, respectively. These results indicate that change of Q252 to G or to N led to an increase in the binding affinity of TRβ1 to F2 of approximately 2.5−3.6-fold.

It is known that F2-bound wTRβ1 homodimers are unstable in the presence of T_3 (*18*, *19*, *22*). To assess whether mutation of Q252 to G or N affected the sensitivity

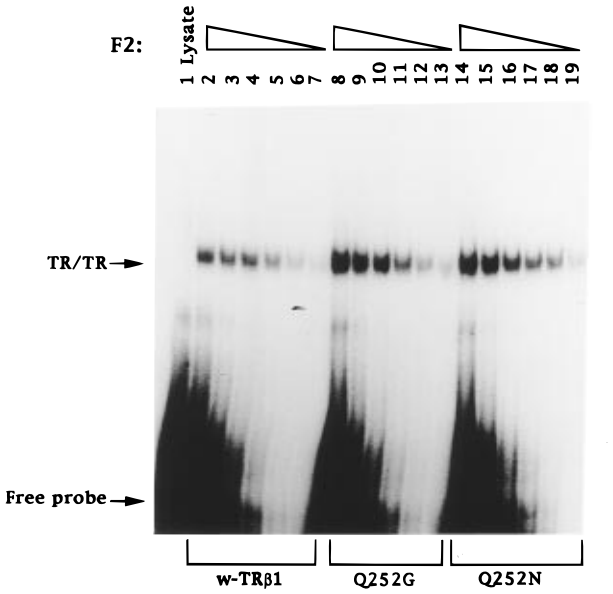

FIGURE 4: Binding of the wild-type and mutant TRβ1 to F2 analyzed by EMSA. Equal amounts of receptors were incubated with increasing concentrations of ^{32}P-labeled F2. The reaction mixture was analyzed by nondenaturing 5% polyacrylamide gel electrophoresis. The F2-bound receptors and the free ^{32}P-labeled F2 were visualized by autoradiography. Lane 1 contains unprogrammed lysate as a control. Lanes 2−7 are wTRβ1, lanes 8−13 are mutant Q252G, and lanes 14−19 are mutant Q252N. The amounts of F2 in each set of lanes were 100, 50, 25, 12.5, 6.2, and 3.1 fmol. The TRE-bound receptors and free TRE were quantified by PhosphoImager.

of F2-bound TRβ1 to T_3, we compared the binding of mutants to F2 in the absence (lanes 6 and 10 of Figure 5A) or presence of T_3 (lanes 7 and 11 of Figure 5A). Similar to wTRβ1 in which no F2-bound wTRβ1 was detected in the presence of T_3 (lanes 3 vs 2 of Figure 5A), homodimers of both mutants were unstable in the presence of T_3, as indicated by virtually complete dissociation of homodimers from F2 (lanes 7 and 11), indicating that mutation of Q252 to G or N did not change the sensitivity of F2-bound TRβ1 to T_3. These observations are consistent with the above results in that the mutation of Q252 to G or to N did not alter the T_3 binding affinity. It is known that TRβ1 also binds to TREs in which the half-site binding motifs are arranged in a direct repeat separated by four nucleotides (DR4) or in a palindrome (Pal). However, the binding of TRβ1 to these two TREs as homodimers is much weaker than to F2 (*18*). Therefore, we cannot conclude if the DR4- or Pal-bound homodimers had similar extents of instability, because under the present experimental conditions, the homodimeric binding of the wTRβ1 and mutants to either TRE was too weak to be detected (lanes 2, 3, 6, 7, 10, and 11 of Figure 5B,C).

The TRE binding activity of wTRβ1 is enhanced by heterodimerization with RXRβ (*23*). We therefore examined if change of Q252 to G or N affected the binding of TRβ1/RXRβ heterodimers to F2. Interestingly, the two mutants bound to all three TREs with enhanced activity as compared to wTRβ1 (lanes 8, 9, 12, and 13 vs lanes 4 and 5 of Figure 5A−C) regardless of whether T_3 was present or not. Quantitation of the bands showed that the enhancement was ~1.4−2.0-fold higher for the binding of mutants to TREs than for wTRβ1.

Transcriptional Activity of TRβ1 Is Altered by Changing Q252 to G or to N. To evaluate the role of Q252 in mediating the transcriptional activity of wTRβ1, we used

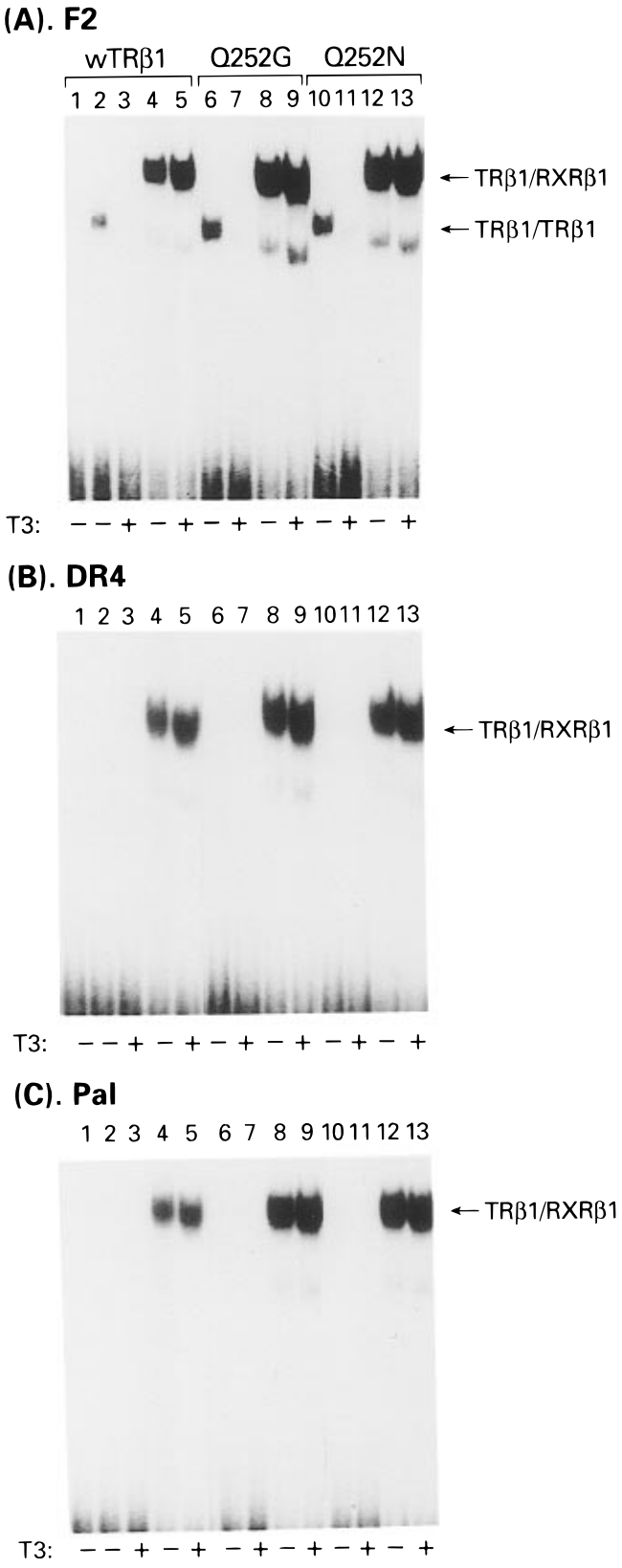

FIGURE 5: Comparison of binding of the RXRβ/wild-type and RXRβ/mutant heterodimers to three TREs in the absence or presence of T_3. The wild-type and mutant receptors were synthesized as described in Materials and Methods. Equal amounts of wTRβ1 (lanes 2−5), mutant Q252G (lanes 6−9), and mutant Q252N (lanes 10−13) were incubated with F2 (A) or DR4 (B) or Pal (C) in the absence of T_3 (lanes 2, 4, 6, 8, 10, and 12) or in the presence of 100 nM T_3 (lanes 3, 5, 7, 9, 11, and 13). In lanes 4, 5, 8, 9, 12, and 13, nuclear extracts (50 ng) containing RXRβ were added. Lane 1 is the lysate control in which only ^{32}P-labeled TRE was present, but no TRβ1 or RXRβ was added.

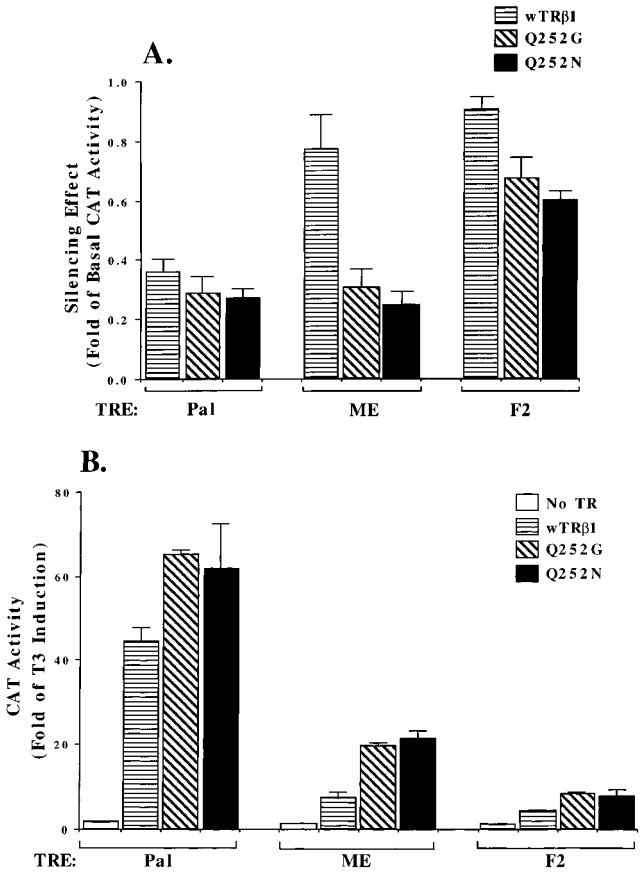

FIGURE 6: (A). Comparison of the silencing activities of wild-type, Q252G, and Q252N TRβ1 mediated by the three TREs. CV1 cells were cotransfected with the reporter plasmid (pTK28mCAT for Pal TRE, pMETKCAT for DR4 or pF2TKCAT; 0.4 μg) and the expression plasmid for wTRβ1 (pCLC51), Q252G (pCLC51-Q252G), or Q252N (pCLC51-Q252N) in the absence of T_3 as described in the Materials and Methods. The CAT activity was normalized to protein concentration of cell lysates. The data are expressed as *x*-fold of the basal CAT activity, which was obtained in the absence of T_3 and the transfected TRs. Data are expressed as mean $\pm$ SD ($n = 8$). (B). Comparison of transcriptional activity of wild-type, Q252G, and Q252N TRβ1 mediated by three TREs. Experimental details are the same as described in panel A except that the activities were determined in the presence of T_3 (100 nM) and the respective TRs. Data are expressed as mean $\pm$ SD ($n = 8$).

transient transfection assays. CV1 cells were cotransfected with wTRβ1 or the mutant TR expression plasmids together with a CAT reporter gene mediated by the three TREs: Pal, ME, and F2. ME is the endogenous TRE from the rat malic enzyme gene which contains the half-site binding motifs arranged as DR4 (*24*, *25*). F2 is the endogenous TRE from the chicken lysozyme gene (*26*). As shown in Figure 6A, in the absence of T_3, wTRβ1 silenced the basal transcriptional activity in the rank order of Pal >ME $\geq$ F2. The mutant TRs were more effective than wTRβ1 in silencing the basal transcriptional activity, showing an increased 25−70% repression with the three TREs. However, as shown in Figure 6B, in the presence of added hormone, the T_3-dependent activated transcriptional activity of mutants Q252G and Q252N was approximately 1.4−2.0-fold higher than that mediated by wTRβ1. The higher transcriptional activity mediated by the mutants was not due to a higher level of expression of TRβ1 proteins. We compared the levels of expression of wTRβ1 and mutants in CV1 cells by Western blotting after cells were transfected with the expression plasmids (Figure 7). We found that the expression levels

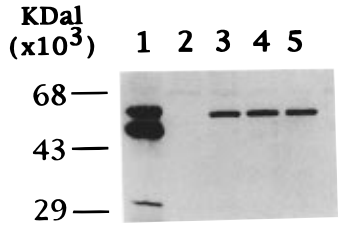

FIGURE 7: *In vivo* protein expression of wTRβ1 and mutants Q252G and Q252N in CV1 cells transfected with expression plasmids. Twenty micrograms of whole cell lysate prepared from the untransfected and transfected CV1 cells as described in Figure 6 were loaded onto an SDS−10% polyacrylamide gel. The resolved proteins were blotted onto a nitrocellulose membrane. The TR proteins were probed with mAb C4, and specific bands were visualized. Lane 2 is the cell lysate from untransfected cells. Lanes 3, 4, and 5 are the cell lysates from cells transfected with expression plasmids for wTRβ1 and mutants Q252G and Q252N, respectively. Lane 1 is from the *in vitro* translated ^{35}S-labeled wTRβ1 used as standard.

of wTRβ1 (lane 3), mutant Q252G (lane 4), and mutant Q252N (lane 5) were essentially identical, indicating that the change of Q252 to G or N did not affect expression at the protein level. The protein bands seen in lanes 3−5 were specific because no TRβ1 band could be detected in the cell lysate prepared from untransfected cells (lane 2). Lane 1 is the positive control using the *in vitro* translated wTRβ1. Taken together, these results indicate that *in vitro* DNA binding activity correlated well with the *in vivo* transcriptional activity of TRs. More importantly, these results further indicate that Q252 is critically involved in the transcriptional activation of TRβ1.

DISCUSSION

Members of the steroid hormone receptor family of homologous transcription factors are made up of four distinct domains, A/B−E, the most conserved of which are the DBD and LBD. The DBD recognizes specific response elements in the DNA sequence, while the LBD, besides binding ligand, participates in transcriptional activation and repression. Hormone binding has been shown to induce conformational changes in receptors, which regulate these properties. The structures of several separate, isolated DNA and ligand binding domains have recently been determined (*7*, *10*, *11*, *27*−*29*), but the structure of any intact receptor and the mechanism of interaction between its structural domains is still unknown.

By mutational analysis, the present study probed the structure in the Ω-loop region of the intact receptor. Movement of the Ω-loop is part of the structural changes of nuclear receptors induced by ligand binding (*8*, *9*). Our studies have yielded important information on the structure and functional role in transcriptional activation of TRβ1 in this region. Previously, we have shown that mAb C3 binds to TRβ1 in the region E248−V256 and is a competitive inhibitor of T_3 binding (*6*). Initially, we interpreted this observation to show that this sequence could be part of the T_3 binding site, a notion which is not supported by the recently determined structure of LBD of rTRα1 (*7*). The result does show, however, that the antibody is specific for the conformation found in apo-TRβ1. Since neither mutant Q252G nor Q252N reacted with mAb C3, we can conclude that they have both undergone a change of conformation in this area. Both mutants showed no impairment in T_3 binding, indicating that this change does not extend to the hormone

binding site. Wagner et al. (*7*) has suggested that this region, together with H1 and H2, forms part of the hinge connecting the LBD to the DBD and may provide a structural means for regulation of DNA binding. In agreement with this, both mutant proteins have increased apparent affinities for the F2, showing a change in interdomain interactions, produced by these substitutions. Using chimeric receptors, we have recently shown that the DNA binding activity of TRs is not entirely dependent on domain C (*30*). Rather, the interaction of domain C with DNA is further fine-tuned by the interplay of domains (*30*). The present findings not only support this notion but also have provided evidence to show that the Ω-loop region could directly or indirectly affect the interaction of DNA domain with DNA.

Apo-TR represses transcription, whereas holo-TR induces transcriptional activation. These functions are believed to be mediated through ligand-dependent interaction with TR-associated proteins (*2*, *31*−*32*). Unliganded TRβ1 has been shown to bind to the corepressor N-CoR in the region E203−V230 (H1) with secondary interactions in the region V230−E260 (H2, Ω-loop) (*33*−*35*). Interestingly, part of the N-CoR binding region, S207−F245, has previously been shown to undergo T_3-induced conformational changes (*5*). Another sequence in this region, P212−I222, has been shown to activate transcription when expressed as a fusion protein with a DNA binding domain (*32*). Thus, hormone-induced conformational changes in the LBD can be transmitted to H1, H2, and the DBD through the extensive movement of the Ω-loop.

Of particular importance were the findings that these two mutants had increased transcriptional activities as compared to wTRβ1. Their more effective silencing and higher responses in T_3-dependent transcriptional activity were due to neither a higher expression of mutant receptor proteins in cells nor a higher T_3 binding affinity. The present data indicate that mutants Q252G and Q252N changed the conformation of the Ω-loop in apo-TRβ1 from that found in wTRβ1, increasing affinity of the aporeceptor for DNA and making this form of the molecule more effective as a repressor, perhaps by transmission of these effects to H1, where N-CoR binds. As discussed above, binding of hormone results in movement of H12 far away from the Ω-loop and exposure of AF-2, for binding of coactivators and negating the repression effect of the mutations on transcription. Our data also indicate that these two mutants bound to RXR with enhanced affinities. Previous reports show that TRs function as heterodimers with RXR (*36*, *37*). Thus, we cannot rule out the possibility that this accounts for their higher transcriptional activities. Our studies clearly indicate that the domains are functionally linked and that Q252 of TRβ1 plays an important role in the transcriptional activation of TRs.

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